Purification and Properties of a Microsomal Enzyme System Catalyzing the Reactivation of Reduced Ribonuclease and Lysozyme

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In a recent report from this laboratory, a rat liver system that catalyzes the conversion of inactive, reduced bovine pancreatic ribonuclease (RNase) to its active form was described (1). This system consists of microsomes and one or more heat-stable, dialyzable components. Previous work on the reactivation of reduced RNase (2, 3) had shown that oxidation of the polypeptide chain of the reduced enzyme to a protein with native conformation and full activity occurs spontaneously, but relatively slowly (even under optimal conditions, *i.e.* pH 8.2, 24°) (4). Reactivation in the presence of the rat liver system, however, proceeds rapidly under more physiological conditions, *i.e.* pH 7.0 to 7.4 and 37°. This latter system has, therefore, been considered a useful model *in vitro* for studying the conversion of polypeptide chains to native proteins. The present paper reports the solubilization and partial purification of the active microsomal protein, and describes some of the properties of the soluble system.

EXPERIMENTAL PROCEDURE

Preparation of Rat Liver Fractions—Homogenates of rat liver were fractionated by differential centrifugation at 0–3° in 0.25 M sucrose-0.01 M Tris-chloride buffer as previously described (1), with a Spinco model L ultracentrifuge. Suspensions of washed microsomes and the 105,000 × *g* supernatant fluid were stored at −20° for as long as 4 months without loss in activity.

Solubilization and Purification of Microsomal Enzyme—A suspension of washed microsomes in 0.25 M sucrose-0.01 M Tris-chloride buffer, pH 7.8, at a protein concentration of 35 mg per ml, was added dropwise, with constant stirring, to 100 volumes of acetone at 0°. The resulting precipitate was collected by filtration and immediately dried in a vacuum. The fluffy acetone powder was homogenized vigorously with a Potter-Elvehjem homogenizer at room temperature in a sufficient amount of 0.2 M NH_4HCO_3 to give a protein concentration of 60 mg per ml. The homogenate was centrifuged at 105,000 × *g* for 10 minutes, and the supernatant fluid was decanted and saved. The residue was extracted a second time, and the combined supernatant fluids were lyophilized. The dry material thus obtained was dissolved in 0.2 M NH_4HCO_3 for further fractionation. Protein concentrations of solutions of microsomal proteins were determined by the biuret method (5), and a Radiometer PHM 22p pH meter was used for pH adjustments.

Gel filtration of the microsomal extract was carried out on several different columns of Sephadex (Pharmacia, Uppsala): G-25, 4 × 45 cm; G-50, 2 × 90 cm; G-100, 2 × 95 cm; and G-200, 4 × 45 cm. In all cases, 0.2 M NH_4HCO_3 was used as the buffer, and pooled fractions were concentrated by lyophilization. All column effluent fractions were monitored by measurement of absorption at 280 mμ with a Beckman DU spectrophotometer.

Fractionation of Supernatant Fluid—The supernatant fluid, prepared by high speed centrifugation of liver homogenates, and referred to below as the "dialyzable fraction," was subjected to gel filtration on Sephadex G-25, and three fractions were obtained as previously described (1). Both the first fraction, containing protein, and the second, which was free of protein, are known to yield a catalytically active system when combined with microsomes.

Reduction of RNase and Lysozyme—Five times recrystallized RNase, type II (Lot RB12-086, Sigma Chemical Company), and twice recrystallized egg white lysozyme (Lot 604, Worthington Biochemical Corporation) were fully reduced with β-mercaptoethanol in 8 M urea, and the reduced proteins were separated from the reagents by gel filtration (2). Chromatography of the RNase on carboxymethyl cellulose (6) revealed that this lot of RNase contained no significant amount of nucleotides, and the presence of approximately 15% of the active RNase "B" component (7) was not considered of importance in the present study. Stock solutions of the reduced enzymes in 0.1 M acetic acid, at concentrations of 0.2 mg per ml (determined spectrophotometrically), were kept at 0° to prevent spontaneous reoxidation.

Reactivation of Reduced RNase—At "zero time," aliquots of the stock solution of reduced RNase were added to solutions containing various combinations of liver fractions and cofactors in Tris-chloride buffer. The incubation mixtures thus obtained had a pH of 7.4, reduced RNase concentration of 0.009 to 0.054 mg per ml, Tris-chloride concentration of 0.09 M, and a final volume of 1.1 or 5.5 ml. The mixtures were agitated in a Dubnoff shaker and maintained at 37°. Assays for RNase activity were performed in duplicate on 0.25-ml aliquots removed from the incubation mixtures immediately after the addition of reduced enzyme and periodically thereafter. The initial rates at which RNase activity appeared (linear for about 3 minutes) were taken as a measure of the reactivating capacities of the mixtures. Specific activities were expressed as the number of millimicro-

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moles of active RNase formed per minute per mg of microsomal protein.

Assay of RNase Activity—RNase activity was assayed by measurement of the rate of digestion of yeast RNA at pH 5.0 (8, 9).

Reactivation of Reduced Lysozyme—At zero time, 0.15 ml of the stock solution of reduced lysozyme was diluted with 0.1 M Tris-chloride buffer to yield a solution with a pH of 7.4, a protein concentration of 0.02 mg per ml, and a volume of 1.5 ml. When rat liver fractions were included in the incubation mixtures, the volume and pH of the buffer were adjusted so that the final volume, pH, and reduced enzyme concentration did not change. Aliquots were removed from the mixtures immediately after addition of reduced enzyme and periodically thereafter, and assayed in duplicate for lysozyme activity. All incubation mixtures were agitated and maintained at 37° in a Dubnoff shaker throughout the reactivation process.

Assay of Lysozyme Activity—Lysozyme activity was assayed turbidometrically in a Cary model 14 spectrophotometer by a modification of the method of Jollès (10, 11), with the use of suspensions of *Micrococcus lysodeikticus* cells (Lot ML C-606-4, Worthington Biochemical Corporation) as substrate. The results are expressed in terms of the percentage of reduced protein converted to active enzyme. Known amounts of native lysozyme, dissolved in the same incubation mixtures (but without reduced lysozyme), were used as standards. The preparation of standards in this manner was important for two reasons: first, the apparent activity of lysozyme is higher in dilute protein solutions than in buffer alone, a fact that is equally true for RNase (1); and second, if reactivation appears to occur more slowly in the presence of a liver fraction than in its absence, it is necessary to distinguish between inhibition of *reactivation* and inhibition of *enzyme activity*.

RESULTS

Purification of Active Protein Component of Microsomes—Experiments demonstrating the marked acceleration of the reactivation of reduced RNase by soluble extracts of acetone powders of microsomes are summarized in Table I. Although there was occasionally a small amount of stimulation induced by the microsomal enzyme alone, the dialyzable fraction of rat liver was always required for maximal effect. No RNase activity appeared when reduced RNase was omitted from the incubation mixtures, indicating that the observed activities truly reflect the conversion of inactive RNase to the active form and not the appearance of a latent RNase activity in the microsomal enzyme preparations.

The active component in microsomal extracts was found to pass unretarded through columns of Sephadex G-25 and G-50, but was retarded when the microsomal extracts were passed through columns of Sephadex G-100 and G-200 (Fig. 1). The G-200 fraction containing material of the highest activity (Fraction 3 in Fig. 1) was lyophilized, and the specific activity of this fraction, determined from the linear portion (the first 3 minutes) of the reactivation curve, was 0.57 mμmole per minute per mg at a substrate concentration of 0.018 mg per ml,[1] representing an 88-fold increase over that of microsomes and a 17-fold increase over that of the microsomal extract. With this material, maximal reactivation (70 to 80%) was achieved in 15 minutes and half-maximal reactivation occurred in less than 3 minutes.

The crude protein fraction (termed Fraction A for convenience) obtained after gel filtration of the microsomal extract on Sephadex G-25 was employed for the study of some of the properties of the reoxidation system. This fraction could be stored indefinitely at −20° without loss of activity. No significant loss of activity occurred on incubation of the fraction at pH 2 and 37° for 15 minutes, whereas heating for 5 minutes at 100° in 0.1 M Tris buffer, pH 7.8, caused virtually complete inactivation.

TABLE I

Reactivation of reduced RNase by soluble extracts of microsomes

Reactivation mixtures containing 0.7 mg of microsomal extract protein, 0.1 ml of the dialyzable fraction, or both, were prepared with a final Tris-chloride buffer concentration of 0.09 M, pH of 7.4, and total volume of 1.1 ml. Except as noted above, reduced RNase (0.02 mg) was added to the mixtures at zero time and the percentage converted to active enzyme in 5 minutes at 37° was determined by assay of aliquots of the mixture for RNase activity. In this series of experiments, no attempt was made to obtain maximal reactivation rates.

Additions to incubation mixture	RNase reactivated in 5 minutes
	%
Microsomal extract	3
Dialyzable fraction	0
Microsomal extract + dialyzable fraction	34
Microsomal extract + dialyzable fraction (reduced RNase omitted)	0

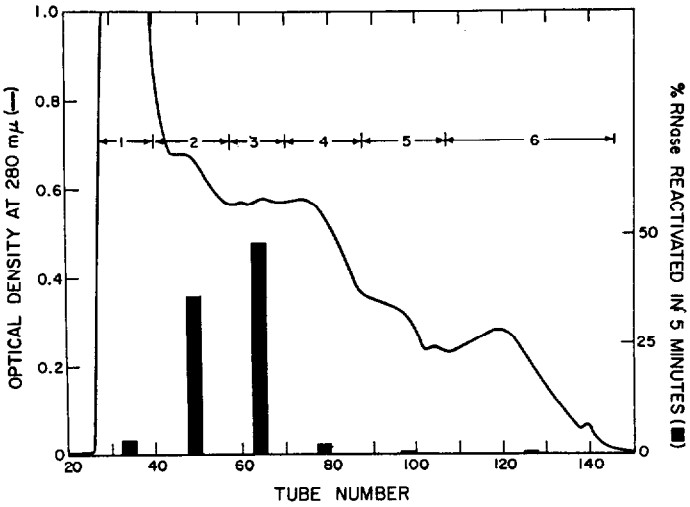

FIG. 1. Gel filtration of the microsomal enzyme. Fraction A, obtained by gel filtration of the microsomal extract on Sephadex G-25, was lyophilized, dissolved in NH_4HCO_3, and applied to a Sephadex G-200 column. The relative activities of the pooled effluent fractions are expressed in terms of their ability to accelerate the reactivation of reduced RNase in incubation mixtures containing the dialyzable fraction, and are represented in the figure by the *solid bars* (0.25 mg of microsomal protein, 0.1 ml of the dialyzable fraction, and 0.02 mg of substrate (reduced RNase) in each incubation mixture; total volume, 1.1 ml).

[1] Since the reoxidation of reduced RNase at high concentrations leads to the formation of inactive intermolecular aggregates (4), the activity of the microsomal enzyme must be determined under conditions in which the substrate, reduced RNase, is rate-limiting (see Table II). Values for the activity of the microsomal enzyme are expressed in this paper in terms of the number of millimicromoles of reduced RNase converted to active enzyme per minute per mg *at an initial substrate concentration of 0.018 mg per ml.*

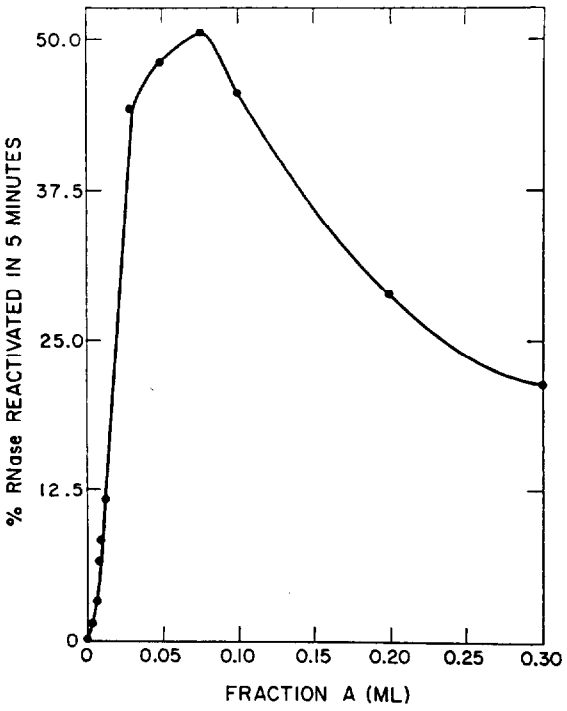

FIG. 2. The effect of different amounts of Fraction A on the initial rate of reactivation of reduced RNase. Reactivation mixtures were prepared with 0.1 ml of the dialyzable fraction and 0 to 0.3 ml of Fraction A (12.7 mg per ml) in a total volume of 1.1 ml (after the addition of reduced RNase). The percentage of the reduced RNase reactivated within 5 minutes is shown as a function of the amount of Fraction A present in the mixtures.

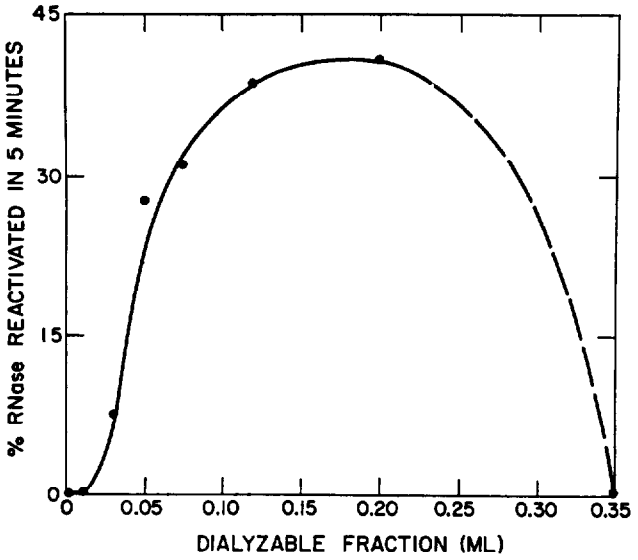

FIG. 3. The effect of different amounts of the dialyzable fraction on the initial rate of reactivation of reduced RNase. Reactivation mixtures were prepared with 0.05 ml of Fraction A (12.7 mg per ml) and 0 to 0.35 ml of the dialyzable fraction in a total volume of 1.1 ml (after the addition of reduced RNase). The percentage of the reduced RNase reactivated within 5 minutes is shown as a function of the amount of dialyzable fraction present in the mixtures.

Kinetics of Reactivation as Function of Concentrations of Reaction Components—When measurements were made of the rate at which reduced RNase was reactivated in incubation mixtures containing 0.1 ml of the dialyzable fraction and 0 to 0.3 ml of Fraction A (12.7 mg per ml) per 1.1 ml of incubation mixture, the results shown in Fig. 2 were obtained. At low concentrations of Fraction A, the increase in the rate of reactivation of reduced RNase was virtually a linear function of concentration. However, as the concentration of this fraction was further increased, a maximum rate was reached, and still higher concentrations produced a marked decrease in the rate of reactivation. Similar results were obtained with the most active fraction (Fraction 3 in Fig. 1) from gel filtration on Sephadex G-200, except that much smaller amounts of protein were required.

The results observed with a set of incubation mixtures in which Fraction A was held constant at 0.05 ml and the dialyzable fraction was varied from 0 to 0.35 ml per 1.1 ml of incubation mixture are shown in Fig. 3. Over this range of concentrations, there was first a rise in the rate of reactivation, then a broad maximum, and, finally, a decrease. The diminution in rate was not due to inhibition of the reoxidized RNase by an inhibitor such as that reported (12) to be present in the supernatant fraction of rat liver homogenates, since the activity of native RNase dissolved in solutions containing from 0.1 to 0.5 ml of the dialyzable fraction per 1.1 ml of total volume did not vary.

When the amounts of both Fraction A and the dialyzable fraction were held constant (at 0.05 and 0.1 ml, respectively, per 1.1 ml of incubation mixture), and the reduced RNase concentration was varied from 0.009 to 0.054 mg per ml, the *initial* (5-minute) yield of RNase activity increased as the RNase concentration was increased (Table II). However, this increase was not strictly proportional to the RNase concentration, and the relative rate of reactivation (*i.e.* the percentage of the total RNase reactivated in the first 5 minutes of incubation) decreased as the RNase concentration was increased.

In Fig. 4 are shown the *final* levels of RNase activity achieved in the first two of the three sets of incubation mixtures described above. In these experiments, in which either Fraction A or the dialyzable fraction was varied, the final yield of RNase activity was constant except at very low or very high concentrations of these fractions. The degree of reactivation represented by the "plateaus" was of the order of 70 to 80% of that theoretically obtainable from the amount of reduced RNase added. When the concentration of the third component of the system, reduced RNase, was varied from 0.01 to 0.04 mg per ml, a constant

TABLE II

Effect of changes in substrate concentration

Incubation mixtures were prepared with 0.05 ml of Fraction A, 0.1 ml of the dialyzable fraction, and various amounts of reduced RNase in a final volume of 1.1 ml. All mixtures, with a pH of 7.4 and a Tris-chloride buffer concentration of 0.09 M, were maintained at 37°. Although the total amount of RNase reactivated in 5 minutes (second column) increased with increasing RNase concentration, the *fraction* of reduced RNase converted to the active form (third column) was inversely related to the RNase concentration.

Concentration of reduced RNase in reactivation mixture	Amount of RNase reactivated in 5 minutes	Fraction of reduced RNase converted to active enzyme in 5 minutes
μg/ml	*μg*	%
9	4.9	54
18	8.5	47
36	9.7	27
54	11.9	22

proportion (approximately 80%) of the reduced protein was eventually converted to active enzyme.

No essential component appears to be used up during the reactivation process. Thus it was observed that after the complete reactivation of one aliquot of reduced RNase, a second aliquot was reactivated at the same rate by the same incubation mixture.

Reactivation of Reduced Lysozyme—The reactivation of reduced lysozyme was completely inhibited in incubation mixtures containing both the purified microsomal enzyme and the dialyzable fraction. This inhibition was found to be due to the dialyzable fraction. Since this material did not inhibit the activity of native lysozyme, its effect must have been due to inhibition of the reactivation process. Indeed, a similar phenomenon was noted during reactivation of reduced RNase but at somewhat higher levels of the dialyzable fraction.

Because of this inhibitory effect, reactivation of reduced lysozyme was attempted in incubation mixtures containing only highly purified microsomal enzyme, and serial assays of lysozyme activity showed that reactivation was greatly accelerated *in the absence of the dialyzable fraction*. In 2 minutes of incubation, 18% of the lysozyme was reactivated while no reactivation occurred in this period of time in the control incubation mixture lacking the microsomal enzyme. When the concentration of the microsomal enzyme was varied over a wide range, results identical with those illustrated for the reactivation of RNase were obtained.

Cofactor Requirements of Microsomal Enzyme—We have shown previously that the reactivation of reduced RNase by liver microsomes required the addition of a supernatant fraction of rat liver (2). The active component or components in this fraction were shown to be stable to heat and acid, to be dialyzable, and to emerge together with salts during gel filtration through Sephadex G-25 columns. As mentioned above, the soluble preparation of the microsomal enzyme has a similar requirement for this fraction (Table I).

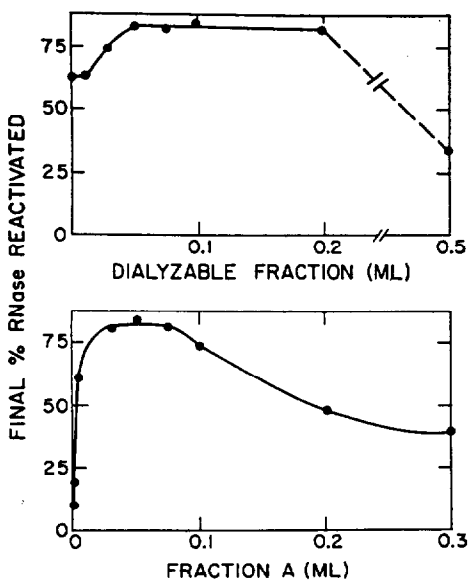

FIG. 4. The effect of various amounts of Fraction A and the dialyzable fraction on the *final extent* of RNase reactivation. *Upper:* the reactivation mixtures were prepared as described in the legend to Fig. 3. *Lower:* the reactivation mixtures were prepared as described in the legend to Fig. 2.

TABLE III

Partial replacement of dialyzable fraction with various cofactors

Incubation mixtures (37°) all had a final Tris-chloride buffer concentration of 0.09 M, pH of 7.4, and volume of 5.5 ml. Where indicated, the mixtures also contained 0.3 ml of a suspension of microsomes (5.0 mg of protein), 0.5 ml of the dialyzable fraction, and various cofactors. In this series of experiments, no attempt was made to obtain maximal rates of reactivation. However, the data provide an indication of the *relative* effectiveness of the various cofactors tested.

Additions to incubation mixture	RNase reactivated in 20 minutes
	%
Microsomes	8
Microsomes + dialyzable fraction	57
Hydroxocobalamin* (10^{-5} M)	0
Microsomes + hydroxocobalamin* (10^{-5} M)	23
GSSG† (10^{-4} M)	0
Microsomes + GSSG (10^{-4} M)	41
FAD*† (10^{-4} M)	0
Microsomes + FAD*† (10^{-4} M)	34

* Hydroxocobalamin was purchased from Squibb Institute for Medical Research. FAD (Lot F26-50) was purchased from Sigma Chemical Company.

† The reactivation process was sensitive to very small changes in the concentration of this cofactor. Only the optimal concentration is shown above.

As shown in Table III, the dialyzable fraction could be partially replaced by hydroxocobalamin and by the oxidized forms of glutathione and flavin adenine dinucleotide when *microsomes* were used.[2] However, neither the initial rate nor the final extent of the reactivation process was as great as when the dialyzable fraction was used. Furthermore, when combined with the soluble and partially purified form of the microsomal enzyme, these substances had little or no effect. A large number of other cofactors of known biological importance were tested and found to be ineffective. In view of the changing cofactor requirements observed during the purification of the enzyme, it is clear that meaningful conclusions on the identity of the active component or components in the dialyzable fraction must await further purification of both protein and nonprotein fractions.

DISCUSSION

When the microsomal system was first investigated, it was not known whether the integrity of the microsome was required. Treatment of microsomes with sodium deoxycholate or acid resulted in loss of activity, but the significance of these observations was uncertain (1). It is clear from the present studies that the integrity of microsomes as organized particles is *not* required for catalyzing the reactivation of the reduced forms of RNase and lysozyme. Large quantities of material of both low and high molecular weight can be removed from soluble extracts of microsomes without loss of activity.

The microsomal enzyme has been purified 88-fold (over microsomes). Considerable purification has also been reported by

[2] Venetianer and Straub (13) have similarly observed that dehydroascorbic acid can replace the heat-stable fraction in their system derived from porcine pancreas for the reoxidation of reduced RNase.

Venetianer and Straub (14) with a similar system derived from chicken pancreas. These authors were able to purify the nondialyzable, heat-labile component of their system 30-fold with respect to an extract of the acetone powder of whole pancreas homogenates by a combination of $(NH_4)_2SO_4$ fractionation and chromatography on DEAE-cellulose. The starting material for the present purification procedures was essentially free of nonmicrosomal proteins.

The availability of a soluble preparation has made it possible to demonstrate that the catalytic reactivation process is virtually first order with respect to the microsomal enzyme. The attainment of a maximal rate at high enzyme concentrations is probably due to a limitation of the quantity of some essential component or components in the system. A likely candidate for this rate-limiting component is the substrate itself, since an increase in the amount of reduced RNase results in an increase in the quantity of RNase reactivated in a given time. The fact that the increase is not strictly proportional to reduced RNase concentration (Table II) may be due to intermolecular aggregation through disulfide bonds (4).

The mechanism of the inhibition by high concentrations of either the dialyzable fraction or the microsomal enzyme is not understood, since it does not appear to be related to inhibition of native RNase. One possibility is that when reactivation is accelerated beyond a certain rate, incorrect pairing of sulfhydryl groups results, leading to the formation of inactive products. Another, more likely possibility is that various impurities in the preparations interact with the reduced protein molecules, preventing reactivation. (For example, it has been observed that small peptides can completely inhibit the reactivation of RNase by the enzyme system.[3]) It is important to note that the *final* yield of RNase activity was independent of the concentration of either the microsomal enzyme or the dialyzable fraction, except at very low or very high concentrations of these fractions. Conversely, when the concentration of reduced RNase was varied, the *final recovery* of RNase activity was directly proportional to the initial concentration of reduced RNase. These findings serve to substantiate the assumption that one is truly measuring the reactivation of reduced RNase, and not the appearance of some latent activity in the liver fractions.

The enzymic nature of the microsomal component is indicated by a number of observations. It is heat-labile and nondialyzable, and it is retarded during gel filtration on Sephadex G-200 but not on G-50. The rate of the reaction that it accelerates is dependent upon its concentration. It accelerates the conversion of more than equimolar amounts[4] of substrate (reduced RNase), indicating that its participation in the reactivation process is not stoichiometric but catalytic.

Although the dialyzable fraction could not be used in the experiments on the reactivation of reduced lysozyme because of its particularly strong inhibitory effect in this system, significant stimulation of the reactivation process could be achieved with the microsomal enzyme alone. A possible explanation is that the enzyme preparation contains a small amount of the required cofactor in bound form.

The observation that the rat liver system can greatly enhance the rate of reactivation of both reduced RNase and reduced lysozyme suggests that the system is nonspecific and may ultimately be shown to be involved generally in the oxidation of the sulfhydryl groups of polypeptides to form the disulfide bonds of the corresponding native proteins.

[3] C. J. Epstein and R. F. Goldberger, unpublished observations.

[4] On the basis of the behavior on Sephadex columns, the assumption is made that the molecular weight of the enzyme is greater than 10,000.

SUMMARY

A soluble protein has been prepared from acetone powders of rat liver microsomes which, when combined with a dialyzable fraction, accelerates the activation of reduced bovine pancreatic ribonuclease. The microsomal protein has been further purified by gel filtration, and the specific activity of the final preparation was 88 times that of microsomes. The oxidized forms of glutathione and flavin adenine dinucleotide, and hydroxocobalamin, can partially replace the dialyzable fraction when whole microsomes are used, but are ineffective with the purified microsomal protein preparation described in this paper. Kinetic studies and calculations of the stoichiometry of the activation process indicate the enzymic nature of the reaction. Activation of reduced egg white lysozyme was also found to be catalyzed by the microsomal protein (in the absence of added dialyzable fraction). The catalysis of the conversion of at least two different polypeptide chains to the corresponding native proteins suggests that the microsomal system may function physiologically to facilitate such a conversion during the synthesis of many proteins containing disulfide bonds.

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